Tonoplast Intrinsic Proteins AtTIP2;1 and AtTIP2;3 Facilitate NH_3 Transport into the Vacuole[1]

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While membrane transporters mediating ammonium uptake across the plasma membrane have been well described at the molecular level, little is known about compartmentation and cellular export of ammonium. (The term ammonium is used to denote both NH_3 and NH_4^+ and chemical symbols are used when specificity is required.) We therefore developed a yeast (*Saccharomyces cerevisiae*) complementation approach and isolated two Arabidopsis (*Arabidopsis thaliana*) genes that conferred tolerance to the toxic ammonium analog methylammonium in yeast. Both genes, *AtTIP2;1* and *AtTIP2;3*, encode aquaporins of the tonoplast intrinsic protein subfamily and transported methylammonium or ammonium in yeast preferentially at high medium pH. *AtTIP2;1* expression in Xenopus oocytes increased ^{14}C-methylammonium accumulation with increasing pH. AtTIP2;1- and AtTIP2;3-mediated methylammonium detoxification in yeast depended on a functional vacuole, which was in agreement with the subcellular localization of green fluorescent protein-fusion proteins on the tonoplast in planta. Transcript levels of both *AtTIPs* were influenced by nitrogen supply but did not follow those of the nitrogen-derepressed ammonium transporter gene *AtAMT1;1*. Transgenic Arabidopsis plants overexpressing *AtTIP2;1* did not show altered ammonium accumulation in roots after ammonium supply, although AtTIP2;1 mRNA levels in wild-type plants were up-regulated under these conditions. This study shows that AtTIP2;1 and AtTIP2;3 can mediate the extracytosolic transport of methyl-NH_2 and NH_3 across the tonoplast membrane and may thus participate in vacuolar ammonium compartmentation.

As a major form of reduced nitrogen, ammonium plays a key role in the metabolism of most cells. (The term ammonium is used to denote both NH_3 and NH_4^+ and chemical symbols are used when specificity is required.) Ammonium is a preferential nitrogen source for many microorganisms and plants. It represents the key compound for the transition of inorganic to organic nitrogen and vice versa, and it is a major form for nitrogen retrieval after unavoidable losses from deamination or transamination processes. Moreover, ammonium triggers the induction of nitrogen assimilatory genes (Ishiyama et al., 2004) and leads to changes in root morphology (Drew, 1975). It is unclear how its external and/or internal concentration is sensed by plant cells, but an NH_4^+ transporter is involved in ammonium sensing in the model eukaryote yeast (*Saccharomyces cerevisiae*; Lorenz and Heitman, 1998).

Cellular uptake of ammonium is mediated by ammonium transporters of the AMT/MEP/Rh protein family, members of which are represented in almost any organism (von Wirén and Merrick, 2004). In plants, members of the AMT subfamily act as NH_4^+ uniporters that transport ammonium along the electrochemical gradient (Ludewig et al., 2002, 2003). Based on an ammonium-induced depolarization of the membrane potential across the plasma membrane of root cells (Shelden et al., 2001), on the plasma membrane localization of two members, LeAMT1;1 and LjAMT2;1 (Ludewig et al., 2003; Simon-Rosin et al., 2003), and on the approximately 30% contribution of AtAMT1;1 to the overall ammonium uptake capacity in Arabidopsis (*Arabidopsis thaliana*) roots (Kaiser et al., 2002), AMT transporters are supposed to play primarily a role in NH_4^+ import into plant cells (Loqué and von Wirén, 2004).

Physiological experiments, however, indicate that ammonium is also exported from the cytoplasm into the apoplasm or into the vacuole: (1) Measuring ammonium concentrations with ion-selective triple-barreled microelectrodes in Chara cells indicated millimolar ammonium concentrations inside the vacuole that largely exceeded those of the cytoplasm (Wells and Miller, 2000); (2) despite large net ammonium uptake, there is considerable efflux from roots preloaded with ^{13}N-labeled ammonium (Britto et al., 2001b); (3) photorespiratory ammonium released in leaf mitochondria require the passage through mitochondrial and chloroplast membranes for reassimilation (Lea and Forde, 1994); and (4) supply of ammonium as a major nitrogen source to rape plants resulted in a large increase of ammonium concentrations in the xylem sap, suggesting that ammonium was exported from pericycle or parenchyma cells for loading of xylem vessels (Finnemann and Schjoerring, 1999). In most cases it is

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unknown if NH_4^+ or NH_3 is the molecular species transported across the corresponding membranes.

With respect to the pK_a of 9.24 for the deprotonation of NH_4^+ to NH_3 and the cytosolic pH of 7.0 to 7.5, approximately 1% of the total cytoplasmic ammonium is present in the uncharged form. Thus, under most physiological conditions, the gradients for NH_3 are directed outward from the cytoplasm, whereas the gradients for NH_4^+ are directed inward (Britto et al., 2001a). The detection of NH_3 emissions from plant leaves clearly supports that a large part of the ammonium losses must occur in the form of NH_3 (Husted and Schjoerring, 1996). Because of the acid pH in the apoplasm, reprotonated apoplastic NH_4^+ can be retrieved efficiently (Nielsen and Schjoerring, 1998).

Besides export of ammonium from the cytoplasm to the apoplasm or to the vacuole, enhanced ammonium assimilation by Gln synthetase is a metabolic process to decrease cytosolic ammonium levels (Oliveira et al., 2002). In plant roots, ammonium assimilation is mainly triggered by a family of cytosolic Gln synthetases, which differ in their biochemical properties as well as in their transcriptional regulation (Ishiyama et al., 2004). If Arabidopsis root cells are excessively supplied with ammonium, a low-affinity Gln synthetase is induced that also exhibits a higher assimilation capacity. Moreover, Glu dehydrogenases that are mainly localized in organelles do also contribute to ammonium detoxification under conditions of excess ammonium supply (Melo-Oliveira et al., 1996).

With the aim of identifying membrane transporters involved in the export of ammonium out of the cytoplasm, a screening approach in yeast was established. To avoid that an enhanced assimilatory capacity may compensate for ammonium toxicity, the substrate analog methylammonium was used, which is hardly converted into methylglutamine in yeast (Soupene et al., 2001). Yeast was transformed with a cDNA library from Arabidopsis, and transformants were screened for tolerance on toxic methylammonium concentrations at high pH. This allowed us to identify two aquaporins of the tonoplast intrinsic protein (TIP) subfamily as exporters for methylammonium. We then employed transport assays in Xenopus oocytes, yeast mutants, and transgenic plants as well as localization studies with green fluorescent protein (GFP)-tagged proteins to further investigate the involvement of the isolated aquaporins in extracytosolic ammonium transport.

RESULTS

AtTIP2;1 and AtTIP2;3 Increase the Tolerance against Toxic Methylammonium Concentrations in Yeast

To isolate plant genes involved in ammonium export or compartmentation, a yeast complementation strategy was designed. To avoid that an enhanced ammonium assimilatory capacity might increase the tolerance of yeast against excess ammonium, ammonium was substituted by the toxic substrate analog methylammonium and supplied to the medium at pH 6.5. With increasing methylammonium concentrations, the yeast wild-type strain BY4741 transformed with the empty vector pDR195 showed severe growth depression and completely failed to grow at 125 mM methylammonium or above (Fig. 1). When the yeast wild-type strain 23344c, which does not require the additional supplement of His, Leu, and Met, was transformed with the empty vector pFL61 or pDR195, it was even more sensitive to methylammonium, resulting in a complete growth depression at 50 mM methylammonium (data not shown). Transformation of 23344c with a cDNA library from Arabidopsis seedlings (Minet et al., 1992) and screening of approximately 400,000 transformants on 50 mM methylammonium allowed us to repeatedly isolate two cDNA clones, *AtTIP2;1* (At3g16240) and *AtTIP2;3* (At5g47450). *AtTIP2;1* encodes a functional δ-TIP tonoplast-located water channel (Daniels et al., 1996) that also transports urea (Liu et al., 2003). The gene product of *AtTIP2;3* is 76% identical to AtTIP2;1 and has not yet been functionally characterized. The exclusive isolation of TIPs suggested that most efficient detoxification of methylammonium by expression of plant genes in yeast is conferred by methylammonium export out of the cytoplasm to the apoplasm or into the vacuole.

Methylammonium Detoxification by AtTIP2;1 and AtTIP2;3 Depends on a Functional Vacuole

To investigate the contribution of the vacuole to methylammonium detoxification in yeast, the vacuole-defective yeast mutant *pep5* was used. While growth of wild-type yeast hardly showed any growth depression at methylammonium concentrations below 20 mM, growth of *pep5* ceased dramatically above 5 mM methylammonium, suggesting that the vacuole increases

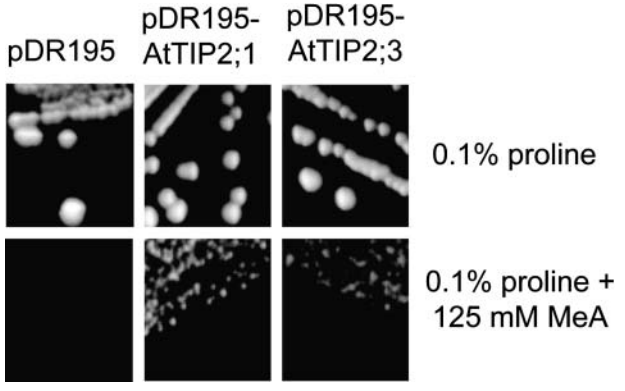

Figure 1. Growth complementation by AtTIP2;1 and AtTIP2;3 of wild-type yeast on toxic methylammonium concentrations. Yeast cells (BY4741) were transformed with pDR195, pDR195-*AtTIP2;1*, or pDR195-*AtTIP2;3* and plated on YNB medium, pH 6.5, supplemented with 0.1% Pro in the presence or absence of 125 mM methylammonium (MeA) and incubated at 28°C for 8 d.

the capacity for methylammonium detoxification in yeast (Figs. 1 and 2). Transformation of *pep5* with *AtTIP2;1* or *AtTIP2;3* did not influence yeast growth relative to transformants with the control vector in the absence of methylammonium. Under supply of 10 to 15 mM methylammonium, however, expression of AtTIPs could not improve methylammonium tolerance in yeast, as it was observed in wild-type yeast (Fig. 1), irrespective of whether cells were grown at pH 6.5 (Fig. 2) or at pH 5.5 (data not shown). This observation supported the notion that both proteins should exert their major function in conferring methylammonium tolerance at the tonoplast.

AtTIP2;1 and AtTIP2;3 Complement Ammonium Uptake in Yeast in a pH-Sensitive Manner

It has been pointed out that heterologous expression of plant membrane proteins in yeast might bear the risk of a mislocalization of the gene product (Bassham and Raikhel, 2000). We tried to take advantage of AtTIP2;1 and AtTIP2;3 gene products that might have been targeted accidentally to the plasma membrane in yeast by assaying their contribution to pH-dependent uptake of external ammonium.

Expression of AtTIP2;1 and AtTIP2;3 in the ammonium uptake-defective yeast strain 31019b, which grows poorly on low ammonium concentrations as a sole nitrogen source, allowed growth complementation even at 1 mM external ammonium, indicating that both AtTIPs conferred substrate transport across the plasma membrane. Growth complementation, however, was observed only at pH 6.5 or 7.5 (Fig. 3). At higher ammonium supply, AtTIP expression further accelerated yeast growth, and growth complementation always increased with pH. Yeast cells transformed with the empty vector also showed better growth at high pH but always lagged behind that of AtTIP-transformed cells. By contrast, ammonium transporter AtAMT1;1-mediated growth complementation was not affected by pH. This experiment suggested that ammonium uptake by both AtTIP proteins mechanistically differs from that of AtAMT1;1 and occurs in the form of NH_3.

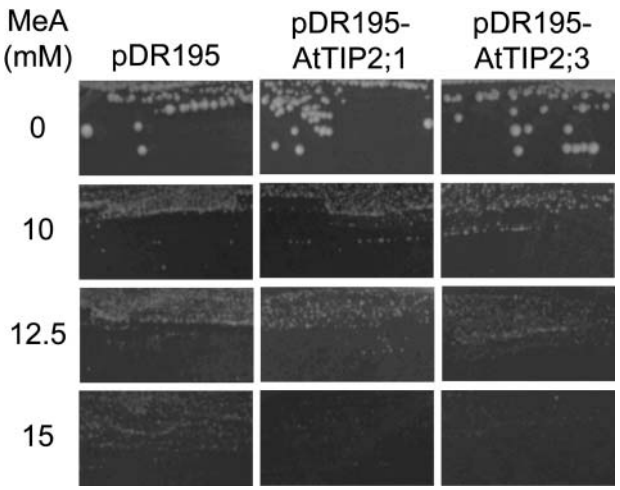

Figure 2. Growth of the vacuole-defective yeast mutant *pep5* in the presence of methylammonium. The yeast mutant *pep5* (Y00817), derived from the wild-type strain BY4741 (Fig. 1), was transformed with pDR195, pDR195-*AtTIP2;1*, or pDR195-*AtTIP2;3*. Transformants were plated on YNB medium, pH 6.5, supplemented with 0.1% Pro in the absence or presence of 10, 12.5, or 15 mM methylammonium (MeA) and incubated at 28°C for 14 d.

Transport of Methylammonium in Oocytes Expressing AtTIP2;1

As an independent approach to verify transport of methylammonium, *AtTIP2;1* was expressed in oocytes, and uptake of ^{14}C-labeled methylammonium was measured in dependence of solution pH. Methylammonium uptake into water-injected oocytes, similar to yeast, increased with increasing pH and a concomitantly increased formation of methyl-NH_2 (Fig. 4). At all pH values tested, oocytes expressing *AtTIP2;1* showed a higher capacity for methylammonium transport, and this transport capacity further increased with pH. Since the concentration of methyl-NH_2, but not of methyl-NH_3^+, increased by a factor of 10 with each pH unit (Fig. 4), AtTIP2;1-mediated substrate transport increased with the availability of the uncharged nitrogen form. Heterologous expression of AtTIP2;1 and AtTIP2;3 in both yeast and oocytes thus indicated that besides water and urea, NH_3 and methyl-NH_2 are novel substrates transported by TIPs.

Tonoplast Localization of AtTIP2;3

Transient expression of AtTIP2;1-GFP fusions in Arabidopsis protoplasts yielded strongest fluorescence at the tonoplast and in small vesicular structures that were released after osmotic shock (Liu et al., 2003). A recent study employing stably overexpressed AtTIP2;1-GFP confirmed tonoplast localization of AtTIP2;1 (Saito et al., 2002). We thus generated transgenic plants for stable expression of AtTIP2;3-enhanced GFP (EGFP) under control of a cauliflower mosaic virus (CaMV) 35S promoter and investigated fluorescence by microscopy. AtTIP2;3-dependent fluorescence appeared in roots as well as in shoot organs but was best visible in rhizodermal cells. AtTIP2;3-EGFP clearly localized on the tonoplast, which has been exemplified by fluorescence around a nucleus (Fig. 5). Thus, AtTIP2;1 and AtTIP2:3 are both primarily localized on the tonoplast in Arabidopsis roots.

The Influence of Nitrogen Supply on *AtTIP2;1* and *AtTIP2;3* Gene Expression

Gene expression of *AtTIP2;1* and *AtTIP2;3* in roots was subjected to a strong diurnal regulation (Fig. 6A). mRNA levels of both genes increased after onset of light, peaked approximately at midday, and declined

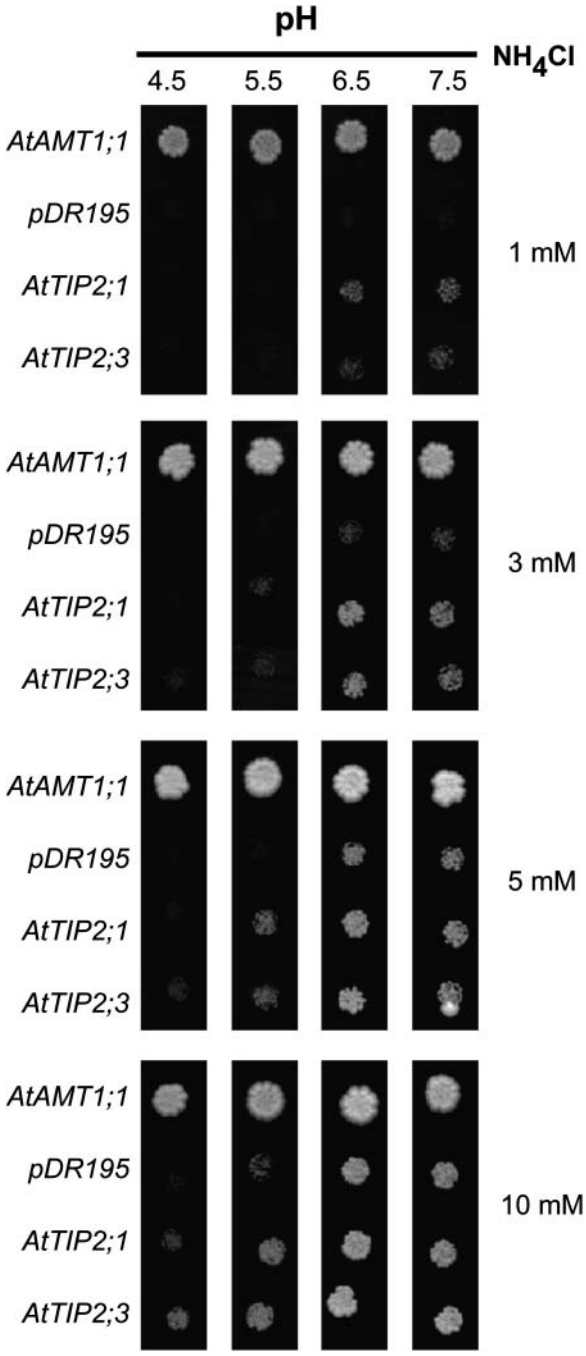

Figure 3. Growth complementation by AtTIP2;1 and AtTIP2;3 of the ammonium uptake-defective yeast strain 31019b. The triple *Mep* yeast mutant (31019b) was transformed with pDR195, pDR195-*AtAMT1;1*, pDR195-*AtTIP2;1*, or pDR195-*AtTIP2;3*. Transformants were precultured for 24 h on YNB medium supplemented with 0.1% Arg. One milliliter of the saturated culture was harvested, resuspended in 1 mL of water, and diluted 1:1,000. Five microliters of diluted cells were spotted on YNB medium supplemented with 1, 3, 5, or 10 mM NH_4Cl at pH 4.5, 5.5, 6.5, or 7.5.

to lowest levels already before offset of light. Such a distinct diurnal regulation is typical for root transporter genes, like those encoding AMT transporters (Fig. 6A; Gazzarrini et al., 1999), that are coregulated by photoassimilates or related metabolites from the shoots (Lejay et al., 2003).

Subjecting hydroponically-grown Arabidopsis plants to nitrogen starvation initially decreased root transcript levels of *AtTIP2;3* (Fig. 6B). *AtTIP2;1* transcript levels oscillated during the experimental period, but for both *AtTIP2;1* and *AtTIP2;3* no monophasic response to nitrogen deficiency was observed. This pattern of gene expression was in agreement with the northern analysis of *AtTIP2;1* in Liu et al. (2003) but clearly different from the pattern observed for the nitrogen-repressed ammonium transporter gene *AtAMT1;1*, for which mRNA levels continuously increased with nitrogen deficiency. The oscillating changes in *AtTIP2;1* and *AtTIP2;3* mRNA levels upon nitrogen starvation may also have been due to other regulatory factors, such as small changes in the osmotic potential of the roots. The distinct regulation from *AtAMT1;1* suggested that induction or derepression of *AtTIP2;1* and *AtTIP2;3* is not a part of the nitrogen-deficiency response in Arabidopsis roots.

In general, gene expression of major intrinsic proteins (MIPs) can be dramatically influenced by the water status of the plant, and ammonium, but in particular nitrate, resupply might lead to a rapid change in root water uptake or in the hydraulic conductivity of roots due to their osmotic action besides their function as a nitrogen source. Moreover, the transfer of plants from nitrogen deficiency to exclusive supply of ammonium or nitrate is also accompanied by secondary effects, such as decreased transpiration rates and xylem loading of cytokinins under ammonium supply (Walch-Liu et al., 2000; Guo et al., 2002). To suppress secondary effects while verifying a

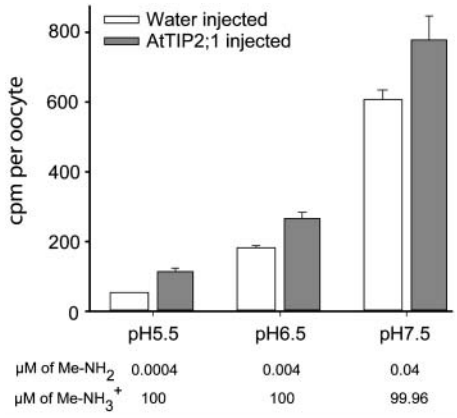

Figure 4. ^{14}C-Methylammonium uptake in oocytes expressing AtTIP2;1. Uptake rates of ^{14}C-methylamonium in noninjected (white bars) and AtTIP2;1-expressing (gray bars) oocytes (n = 3–4). Uptake assays were conducted in HEPES-buffered solution at pH 5.5, 6.5, and 7.5 under supply of 100 μM external methylammonium.

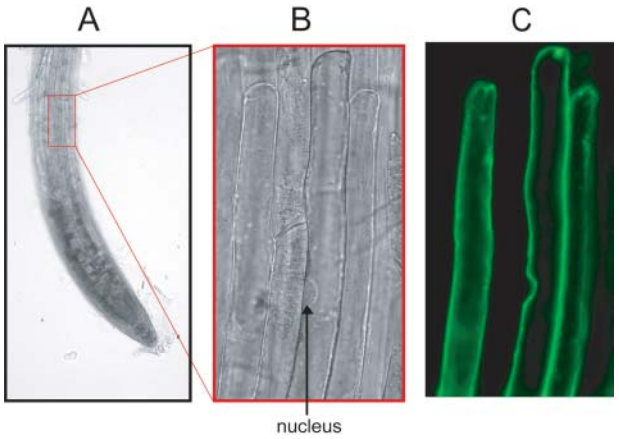

Figure 5. Subcellular localization of AtTIP2;3-EGFP fusion proteins. Transformed plants were grown on half-strength Murashige and Skoog medium supplemented with 1% Suc for 1 week before image analysis using inverted fluorescence microscopy. A, Root tip (20×/0.60) by transmission imaging. B, Epidermal root cells (63×/1.4) by transmission imaging. C, Epidermal root cells (63×/1.4) by fluorescence imaging of Arabidopsis plants transformed with CaMV35S-AtTIP2;3-EGFP.

dependence of *AtTIP* gene expression on ammonium supply, plants were transferred from 5 mM nitrate to 4 mM nitrate plus 1 mM ammonium. Under these conditions *AtTIP2;1* and *AtTIP2;3* clearly responded to ammonium supply by a dramatic increase in transcript levels within 24 h (Fig. 7A). Since this expression pattern might indicate a role in ammonium loading into the vacuole, we simultaneously determined changes in ammonium concentrations in the roots and found that they increased rapidly after plant transfer to ammonium, peaked after 3 h, and then slightly declined (Fig. 7B). In particular *AtTIP2;1* transcript levels increased in parallel to ammonium concentrations in the root tissue until ammonium concentrations reached their maximal level. Again, this regulation differed to that of *AtAMT1;1* and indicated that *AtTIP2;1*, and probably also *AtTIP2;3*, may participate in vacuolar compartmentation and detoxification of ammonium.

Overexpression of *AtTIP2;1* in Arabidopsis Does Not Enhance Short-Term Ammonium Accumulation

To verify an involvement of AtTIP2;1 in vacuolar NH_3 loading in Arabidopsis roots, *AtTIP2;1* was overexpressed under the control of a CaMV *35S* promoter in Arabidopsis plants. Four homozygous lines were selected that showed dramatically increased expression levels in the roots relative to the wild type (Fig. 8A). When grown under adequate supply of nutrients or under nitrogen deficiency in nutrient solution, transgenic plants did not show any visible phenotype. Moreover, *AtTIP2;1*-overexpressing lines were not more tolerant to methylammonium at pH 6 when grown on agar (data not shown). We therefore investigated whether the overexpression of *AtTIP2;1* might increase ammonium accumulation in roots under conditions of ammonium induction of nitrate-fed plants. Ammonium concentrations in roots of *AtTIP2;1* overexpressors, however, were not different from those of wild-type plants either under nitrate nutrition, after supply of 1 mM ammonium for 3 h (Fig. 8B), or for longer and shorter periods (data not shown). Since ammonium accumulation in the root tissue was rapid, ammonium transport across the plasma membrane was unlikely to represent a limiting step for subsequent vacuolar compartmentation, irrespective of whether uptake was mediated by transporters with high affinity, such as AtAMTs, or by transporters with low affinity, which are not yet identified. Thus, under these conditions, constitutive overexpression could not demonstrate an involvement of AtTIP2;1 in methylammonium detoxification or in ammonium accumulation in roots.

DISCUSSION

AtTIP2;1 and AtTIP2;3 Transport NH_3

Although physiological studies indicated that extracytosolic transport of ammonium is involved in several processes, such as vacuolar ammonium loading, ammonium efflux from roots, or xylem loading, the molecular basis for extracytosolic ammonium transport remained unclear. This study shows that extracytosolic transport of ammonium and, thus,

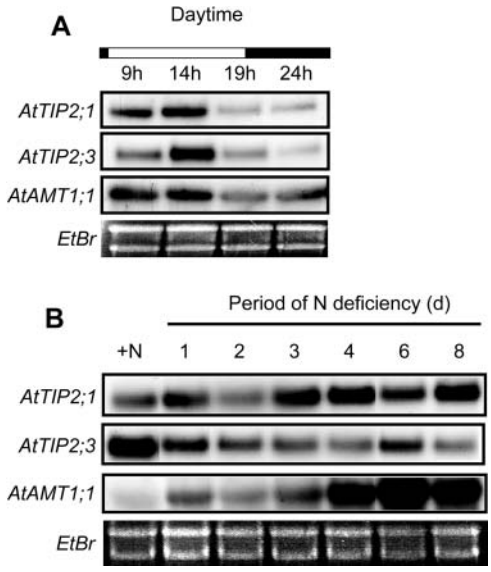

Figure 6. Diurnal rhythm and nitrogen dependency of *AtTIP2;1* and *AtTIP2;3* gene expression. A, RNA gel-blot analysis of *AtTIP2;1*, *AtTIP2;3*, and *AtAMT1;1* expression at different times of day. Plants were grown for 5 weeks in hydroponic culture with supply of 2 mM NH_4NO_3 and harvested at 9 h (onset of light), 14 h (middle of light period), 19 h (offset of light), and 24 h (dark). B, RNA gel-blot analysis of *AtTIP2;1*, *AtTIP2;3*, and *AtAMT1;1* expression in roots from 6-week-old plants grown in hydroponic culture with 2 mM NH_4NO_3 before nitrogen starvation for 1 to 8 d. All plants were harvested at the same time of day.

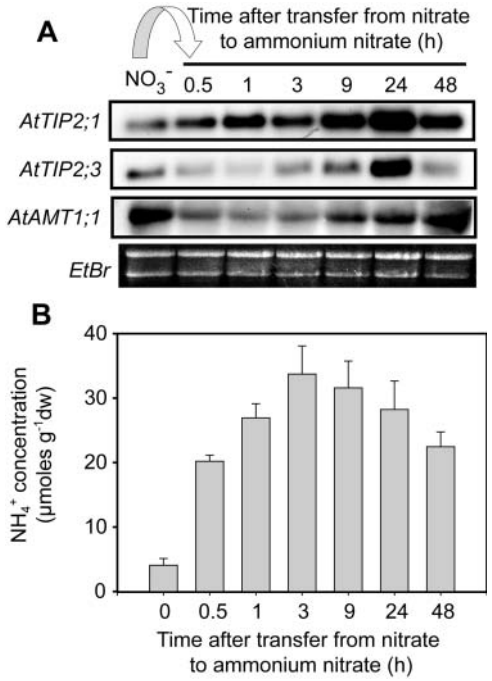

Figure 7. *AtTIP2;1* and *AtTIP2;3* gene expression in ammonium-induced Arabidopsis roots. A and B, RNA gel-blot analysis of *AtTIP2;1*, *AtTIP2;3*, and *AtAMT1;1* expression (A) and ammonium concentrations in roots (B) of 6-week-old plants grown in hydroponic culture with 5 mM nitrate before transfer to 4 mM nitrate supplemented with 1 mM ammonium for 0.5 to 48 h. All plants were harvested at the same time of day.

cytosolic detoxification can be mediated in the form of NH_3 by water channels of the TIP subfamily. The accumulation of ammonium in the acidic vacuolar lumen is best explained by an acid-trap mechanism, in which the NH_3 diffusing across the tonoplast subsequently binds a proton to form NH_4^+. Three independent methodological approaches provided evidence that the two TIPs AtTIP2;1 and AtTIP2;3 mediate the transport of NH_3: (1) Heterologous expression of both transporters in wild-type yeast resulted in an enhanced tolerance against methylammonium, a toxic analog to ammonium; (2) expression of both AtTIPs in the ammonium uptake-defective yeast strain 31019b conferred yeast growth through ammonium uptake; and (3) expression of AtTIP2;1 in oocytes accelerated the transport of methylammonium. These approaches clearly depended on external pH in a way that methylammonium or ammonium transport increased with increasing pH and, thus, with an increasing formation of methyl-NH_2 or NH_3. Since the protein activities of AtTIPs are insensitive to external pH (Liu et al., 2003; Tournaire-Roux et al., 2003), these observations allowed us to conclude that AtTIP2;1 and AtTIP2;3 mediate the transport of NH_3.

NH_3 permeation by plant aquaporins was first predicted from experiments on peribacteroid membranes showing that the permeation of NH_3 can be inhibited by mercury or silver, which are potent inhibitors of a large range of aquaporins (Preston et al., 1993; Daniels et al., 1996; Niemietz and Tyerman, 2000, 2002). Transport of NH_3 by members of the TIP subfamily of aquaporins (Fig. 3) is not surprising with regard to the recent structural classification of Arabidopsis MIPs. Based on the x-ray structural analysis of the mammalian aquaporin AQP1 and the *Escherichia coli* glyceroporin GlpF, homology modeling and sequence alignments were performed by Wallace and Roberts (2004) to subdivide Arabidopsis MIPs into eight subclasses according to their amino acid signatures in the predicted selectivity filter, which is represented by the narrowest constriction region in the pore of these proteins. Notably, AtTIP2;1 and AtTIP2;3 belong to the same subgroup, which is characterized by a hydrophilic His residue, instead of a typical hydrophobic aromatic residue, located in the second and a conserved aliphatic residue (Ile) located in the fifth transmembrane-spanning domain adjacent to the constriction region. Relative to plasma membrane intrinsic protein-type aquaporins, this amino acid signature leads to a reversed polarity within the constriction region of AtTIP2;1 and AtTIP2;3 (Wallace and Roberts, 2004), which might favor the transport of NH_3. Homology modeling and the identification of AtTIP2;1 and AtTIP2;3 as NH_3 transporters alone, however, did not allow us to conclude on the substrate selectivity of plant MIPs in general. First, employing AQP1 as a water-selective aquaporin for homology modeling (Wallace and Roberts, 2004) is not in agreement with

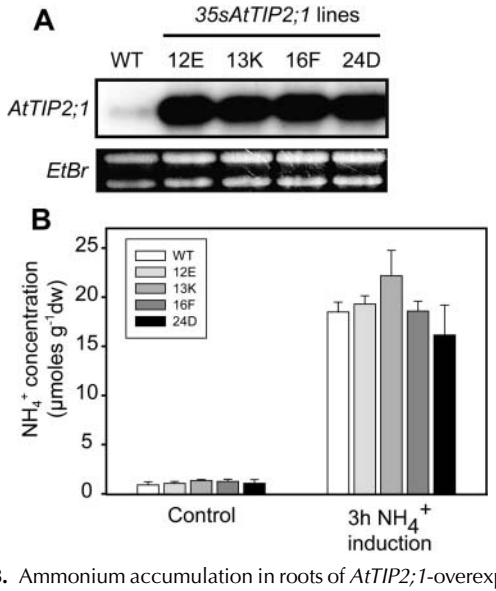

Figure 8. Ammonium accumulation in roots of *AtTIP2;1*-overexpressing Arabidopsis lines. A, RNA gel-blot analysis of *AtTIP2;1* expression in wild-type (WT) and four independent *AtTIP2;1*-overexpressing lines (12E, 13K, 16F, and 24D) of 6-week-old plants grown in hydroponic culture with 5 mM nitrate. B, Ammonium concentrations in wild-type (WT) and *AtTIP2;1*-overexpressing lines from 6-week-old plants grown in hydroponic culture with 5 mM nitrate or 3 h after transfer to 4 mM nitrate supplemented with 1 mM ammonium. All plants were harvested at the same time of day.

an additional NH_3 transport function as proposed by Nakhoul et al. (2001). Second, MIPs other than the two isolated TIPs might not have been isolated due to a lower abundance of their cDNAs in the library or due to a less efficient translation and tonoplast targeting in yeast.

Due to the dissociation of NH_4^+ into NH_3 and H^+ with a pK_a of 9.24, it is impossible to supply NH_4^+ or NH_3 as a sole substrate at physiological pH. In our heterologous expression studies, we thus successively increased pH to raise the concentration ratio of the noncharged substrate species relative to the charged species. In the yeast and oocyte assays, the ammonium or methylammonium transport function of AtTIP2;1 and AtTIP2;3 increased with an increasing formation of NH_3 or methyl-NH_2 (Figs. 3 and 4). This is in contrast to the behavior of AMT-type ammonium transporters like AtAMT1;1, LeAMT1;1, and LeAMT1;2, for which the NH_4^+-transport capacity was not affected when the external pH was shifted from pH 5 to 8 (Fig. 3; Ludewig et al., 2002, 2003). Further support of an NH_3 transport function by aquaporins has been obtained from transport studies with oocytes expressing the human aquaporin AQP1. In that study, ammonium supply progressively stimulated internal alkalinization of the oocyte with increasing external pH, without, however, affecting the membrane potential. This is best explained by the transport of NH_3 and substrate protonation inside the oocyte (Nakhoul et al., 2001).

Our observations suggested that a physiological function of AtTIP-mediated NH_3 transport in planta depends not only on compartmental pH and a concentration gradient along the transport direction, but also on the intrinsic NH_3 permeability of the membrane lipid bilayer (Lande et al., 1995). To our knowledge, NH_3 permeabilities in dependence of lipid composition or MIP densities have not yet been described for different plant membranes, in contrast with permeabilities for the nonelectrolyte urea, which is also a substrate for AtTIP2;1 (Liu et al., 2003) and for AtTIP2;3 (data not shown). Tonoplast-enriched vesicles from wheat (*Triticum aestivum*) showed a high, mercury-sensitive urea permeability in contrast with the lower permeability of plasma membrane-enriched vesicles that was mercury insensitive (Tyerman et al., 1999). Likewise, using pH-sensitive fluorescence to monitor ammonia permeability through peribacteroid membrane vesicles allowed the demonstration that the NH_3 permeability is strongly reduced in the presence of mercury, thereby being as similarly sensitive as aquaporin-mediated water permeation (Niemietz and Tyerman, 2000). Since nodulin-26, a member of the NOD26-like intrinsic protein (NIP) subfamily of MIPs, represents a major component of peribacteroid membranes, it was suggested that either nodulin-26 itself or other mercury-sensitive membrane proteins may permeate NH_3 (Niemietz and Tyerman, 2000). Taken together, these studies support our conclusion that AtTIP2;1 and AtTIP2;3 are able to transport NH_3 in addition to water.

AtTIP2;1 and AtTIP2;3 Mediate Substrate Transport across the Tonoplast

Previous studies clearly showed that AtTIP2;1 is mainly localized in the tonoplast. Western-blot analysis detected the AtTIP2;1 protein mainly in membrane protein fractions from isolated Arabidopsis vacuoles (Daniels et al., 1996). More recently, transient expression of a GFP-tagged fusion protein resulted in AtTIP2;1-mediated fluorescence of the tonoplast as well as of vesicular structures (Liu et al., 2003). These structures have been identified as bulbs representing invaginations of the tonoplast, and stable expression of AtTIP2;1-GFP in transgenic Arabidopsis confirmed targeting of AtTIP2;1 to the tonoplast (Saito et al., 2002). Overexpression of AtTIP2;3-EGFP fusion proteins in transgenic Arabidopsis plants also demonstrated tonoplast localization (Fig. 5), similar to AtTIP2;1. Thus, both proteins might fulfill a similar cellular transport function.

If AtTIP2;1 and AtTIP2;3 were methyl-NH_2- and NH_3-transporting tonoplast proteins, their action should be dependent on a functional vacuole. To test this hypothesis, we expressed both proteins in the vacuole-defective yeast strain *pep5* and investigated their contribution to methylammonium detoxification. Indeed, both proteins were unable to improve tolerance to toxic methylammonium concentrations (Fig. 2). Together with the observation of a higher sensitivity of *pep5* cells to methylammonium relative to their isogenic wild type (Fig. 1), this experiment indicated that the vacuole itself as well as AtTIP-mediated transport across the tonoplast play an important role in vacuolar loading of methylammonium and most likely also of ammonium. Aquaporin-mediated NH_3 transport could also explain the vacuolar alkalization in response to ammonium supply at high pH as determined by ratiometric pH measurements in root hairs of maize (*Zea mays*) and rice (*Oryza sativa*) employing pH-sensitive fluorescent dyes (Wilson et al., 1998). With regard to different physiological functions related to different types of vacuoles and the possibility of employing TIP isoforms for the identification of different types of vacuoles (Jauh et al., 1998, 1999), it will be interesting in the future to correlate the substrate specificity of individual AtTIPs with their intracellular localization to verify whether NH_3-transporting TIPs are related to specialized vacuolar functions.

AtTIP2;1 and *AtTIP2;3* Gene Expression Responds to Ammonium Supply

To identify if the contribution of AtTIP2;1 and AtTIP2;3 in NH_3 loading of vacuoles was physiologically regulated, a series of gene expression analyses was conducted aimed at identifying a possible regulatory link between *AtTIP* gene expression and the nitrogen nutritional status of the plant. In nitrogen-deficient growth conditions, cytosolic ammonium concentrations were low, suggesting that vacuolar

remobilization of ammonium should dominate over vacuolar loading (Fig. 7B). Indeed, in contrast with *AtAMT1;1*, mRNA levels of *AtTIP2;3* were down-regulated under prolonged nitrogen deficiency, while those of *AtTIP2;1* oscillated (Fig. 6B). With respect to the observation that nitrogen deficiency leads to a major decrease in root hydraulic conductivity (Clarkson et al., 2000), mRNA levels might not have responded exclusively to nitrogen deficiency. Since *AtTIPs* are regulated at the transcript level by different environmental cues and in particular by changes in the osmotic potential (Kjellbom et al., 1999; Tyerman et al., 2002), plants were transferred from nitrate-containing solution to nitrate solution supplemented with ammonium, which then caused a dramatic up-regulation of both *AtTIP2;1* and *AtTIP2;3*, while *AtAMT1;1* mRNA levels remained almost stable (Fig. 7A). This ammonium-induced gene expression, as has also been reported for mammalian aquaporins (Rama Rao et al., 2003), clearly supports a function of the corresponding proteins in ammonium detoxification of the cytosol and/or vacuolar ammonium storage under conditions when tissue concentrations rapidly increase (Fig. 7B). In contrast with *AtAMT1;1*, the transcriptional activation of *AtTIP2;1* and *AtTIP2;3* was atypical for a nitrogen deficiency stress response, further supporting that their protein functions are not primarily related to cellular ammonium acquisition but rather to a downstream process. All nitrogen-dependent changes in gene expression, however, were additionally subject to an underlying oscillation. Such oscillations in MIP gene expression have been described as resulting from diurnal fluctuations in root hydraulic conductivity (Clarkson et al., 2000; Tyerman et al., 2002) that integrate over several different growth factors and thus may partially dominate over a nitrogen-dependent regulation.

The Physiological Significance of AtTIP2;1- and AtTIP2;3-Mediated NH_3 Transport

Assigning a physiological function based on gene regulation and permeability characteristics alone would disregard the possibility that NH_3 transport by TIP proteins might just represent a nonspecific side activity of this transporter class. Following the rationale that a higher density of TIPs at the tonoplast might accelerate NH_3 loading of vacuoles, Arabidopsis plants were generated with strongly increased levels of *AtTIP2;1* mRNA levels (Fig. 8A). Since the transfer of wild-type Arabidopsis plants from nitrate- to ammonium nitrate-containing nutrient solution caused a rapid increase in ammonium concentrations in roots (Fig. 7B), transgenic plants were assayed already 3 h after transfer when gene expression levels in wild-type plants were less up-regulated (Fig. 7A). However, after 3 h of incubation, total ammonium concentrations in transgenic lines overexpressing *AtTIP2;1* did not deviate from the wild type (Fig. 8B). Thus, the capacity or the rate of ammonium accumulation in root cells, a process that should involve vacuolar loading, could not be increased by *AtTIP2;1* overexpression. The failure to demonstrate AtTIP2;1 functionality in this approach might have several causes. (1) Enhanced mRNA levels of *AtTIP2;1* might have been compensated for by posttranscriptional down-regulation; indeed, posttranscriptional regulation of tonoplast aquaporins has been indicated in radish (Suga et al., 2001). Moreover, MIP activity has been shown to be controlled by phosphorylation (Maurel et al., 1995; Johansson et al., 1998; Niemietz and Tyerman, 2000), and AtTIP2;1 also exposes putative phosphorylation sites to the cytoplasm. (2) Theammonium concentration in roots might not reflect ammonium accumulation in the vacuole and is thus not a sensitive parameter for AtTIP activity in the tonoplast; therefore, future experiments will aim at determining ammonium concentrations in isolated vacuoles. Or (3), there is already a high density of AtTIPs in the tonoplast of wild-type plants (Karlsson et al., 2000), so that *AtTIP2;1*-overexpression could not further increase the rate of NH_3 transport across the tonoplast.

Although this study could not yet demonstrate unequivocally the physiological significance of AtTIP-mediated NH_3 transport in planta, it did show that AtTIP2;1 and AtTIP2;3 transport ammonium and methylammonium efficiently only at high medium pH and thus most likely represent NH_3 transporters. With their localization in the tonoplast and their transcriptional activation under ammonium supply, they are the most promising candidates to participate in the vacuolar loading of NH_3, thereby accumulating ammonium to concentrations above those of the cytoplasm. In the future, a more refined analysis of transgenic plants overexpressing one of these AtTIPs will have to consider not only the targeting and posttranscriptional regulation of overexpressed AtTIPs, but also the cell type-specific expression of the endogenous AtTIPs and the determination of subcellular NH_3 transport with more sensitive tools.

MATERIALS AND METHODS

Screening of Transformed Yeast Cells for Tolerance against Methylammonium Toxicity

The *ura*$^-$ yeast (*Saccharomyces cerevisiae*) wild-type strain 23344c (Marini et al., 1997) was transformed with a cDNA library from Arabidopsis (*Arabidopsis thaliana*) seedlings (Minet et al., 1992) and directly selected on solid yeast nitrogen base (YNB) medium supplemented with 2% Glc, 0.1% Pro, 50 mM methylammonium, buffered at pH 6.5 by 5 mM MES-Tris. Open reading frames (ORFs) of the isolated cDNAs *AtTIP2;1* (At3g16240) and *AtTIP2;3* (At5g47450) were amplified by PCR using Pfu Turbo DNA polymerase (Stratagene, La Jolla, CA). PCR products were A-tailed with Taq-polymerase, ligated into the pGEM-T Easy vector (Promega, Madison, WI), and subcloned into the yeast expression vector pDR195 (Rentsch et al., 1995) using *Not*I sites and reintroduced into the wild-type yeast strain.

Yeast Complementation on Methylammonium and Ammonium

For growth complementation, the yeast strains *pep5* Y00817 (BY4741; Mat a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YMR231w::kanMX4), its isogenic wild-type

BY4741 (Mat a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0), both from Euroscarf (http://www.uni-frankfurt.de/fb15/mikro/euroscarf/), and the triple *mep* deletion strain 31019b (Δ*mep1;2;3*; Marini et al., 1997) were used. The complementation assays on methylammonium were performed on solid YNB medium (minimal yeast medium without nitrogen) supplemented with 3% Glc, 0.1% Pro, and different amounts of methylammonium, buffered at pH 6.5 by 50 mM MES-Tris. The pH-dependent growth complementation on ammonium was conducted on solid YNB medium supplemented with 3% Glc, 1, 3, 5, or 10 mM ammonium chloride, 100 mM potassium chloride and buffered at different pH by 50 mM MES-Tris.

Radiotracer Uptake Studies in *Xenopus laevis* Oocytes

The ORF of *AtTIP2;1* was subcloned from the pGEM-T Easy vector using *Eco*RI cutting sites into the oocyte expression vector pOO2 (Ludewig et al., 2002). Capped cRNA was transcribed from pOO2-*AtTIP2;1* in vitro using the mMessage kit (Ambion, Austin, TX) after linearization with *Mlu*I, respectively. *Xenopus laevis* oocytes were removed from adult female frogs by surgery and manually dissected. Oocytes (Dumont stage V or VI) were defolliculated using 10 mg mL^{-1} collagenase (Boehringer, Mannheim, Germany) and trypsin inhibitor (Sigma-Aldrich, St. Louis) for 1 h and injected with ≈5 nL of cRNA (5 ng per oocyte). Oocytes were kept after injection for 3 d at 16°C in ND96 solution of the following composition: 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM 2-HEPES, supplemented with 2.5 mM sodium pyruvate and 20 μg mL^{-1} gentamycin, pH 7.4.

Standard bath solutions were used for uptake experiments. Oocytes were pooled in groups of four and incubated for 20 min at room temperature in 500 μL of the appropriate buffer containing 100 μM methylammonium labeled with 10% ^{14}C-labeled methylammonium (specific activity of 57 mCi/mmol; Amersham, Buckinghamshire, UK). Then, oocytes were washed carefully five times in 1 mL of ice-cold buffer with 100-fold excess of cold methylammonium and solubilized in 5% SDS. After the addition of 5 mL scintillation cocktail (Ultima Gold; Zinsser Analytic, Frankfurt), radioactivity was determined in a scintillation counter (Perkin-Elmer, Boston).

Subcellular Localization of AtTIP2;3

The ORF of *AtTIP2;3* was amplified without stop codon using Pfu turbo polymerase. A *Nco*I site was inserted in the reverse primer to ligate in-frame *AtTIP2;3* with the EGFP coding sequence. The DNA coding sequence of AtTIP2;3-EGFP was sequenced and subcloned into the pPPT-kan vector kindly provided by Dr. Karin Schumacher (Zentrum für Molekularbiologie der Pflanzen, Tuebingen, Germany) to generate 35S-AtTIP2;3-EGFP. Transgenic Arabidopsis plants (ecotype Columbia-0) were generated by floral dipping according to Clough and Bent (1998) using *Agrobacterium tumefaciens* GV3101. Independent kanamycin-resistant plants (T0) were isolated and amplified. Localization experiments were conducted with homozygote T2 plants using an inverted fluorescence microscope (Zeiss Axiovert 200M; Jena, Germany).

Generation of *AtTIP2;1*-Overexpressing Arabidopsis Plants

Using an *Eco*RI restriction site, the ORF of *AtTIP2;1* in pGEM-T Easy was subcloned into pGreen0029 (Hellens et al., 2000) containing a p35CaMV-mcs-tCaMV cassette to generate *35S-AtTIP2;1*. Independent Basta-resistant lines (T0) were isolated and amplified. Experiments were conducted with homozygote T2 plants.

Plant Culture

Arabidopsis seeds (ecotype Columbia-0) were germinated in the dark for 4 d and precultured on rockwool moistened with tap water. After 1 week, tap water was substituted by full nutrient solution containing 1 mM KH_2PO_4, 1 mM $MgSO_4$, 250 μM K_2SO_4, 250 μM $CaCl_2$, 100 μM Na-Fe-EDTA, 50 μM KCl, 50 μM H_3BO_3, 5 μM $MnSO_4$, 1 μM $ZnSO_4$, 1 μM $CuSO_4$, and 0.7 μMm $NaMoO_4$, pH adjusted to 6.0 by KOH. Nitrogen was supplied as NH_4NO_3, NH_4Cl, or KNO_3. The nutrient solution was renewed once a week during the first 3 weeks, twice in the 4th week, and every 2 d for the following weeks. Plants were grown hydroponically under nonsterile conditions in a growth cabinet under the following conditions: 10/14 h light/dark; light intensity 280 μmol m^{-2} s^{-1}; temperature 22°C/18°C; and 70% humidity. If not indicated otherwise, plants of the same age were harvested at the same time of day (usually 5 h after onset of light).

RNA Gel-Blot Analysis

Total RNA was isolated by phenol-guanidine extraction followed by lithium chloride precipitation according to Lobreaux et al. (1992). RNA (20 μg per lane) was resolved by electrophoresis on MOPS-formaldehyde agarose gels, blotted onto Hybond-N+ nylon membranes (Amersham), and cross-linked to the membrane by incubation at 80°C for 2 h. The ORFs of *AtTIP2;1*, *AtTIP2;3*, and *AtAMT1;1* were used as a probe for hybridization to total RNA. Hybridization to a randomly primed ^{32}P-radiolabeled probe was performed at 42°C in 50% (v/v) formamide, 1% (w/v) sarkosyl, 5× SSC, and 100 μg mL^{-1} yeast t-RNA. Membranes were washed repeatedly in 2× SSC, 0.1% (w/v) SDS and finally in 0.2× SSC, 0.1% (w/v) SDS at 42°C for 40 min. Ethidium bromide-stained gels were used as RNA loading control.

Ammonium Determination

Before harvest, roots were rinsed in 1 mM $CaSO_4$ solution for 1 min. Root and shoot organs of each plant were separated, immediately frozen in liquid nitrogen, and stored at −70°C before freeze drying. Each sample (25–50 mg) was ground and homogenized with 1 mL of cold 10 mM formic acid solution. After centrifugation at 13,000*g*, the liquid phase was transferred to a new Eppendorf tube and kept on ice. Ammonium concentrations were determined by fluorescence spectroscopy at neutral pH (Husted et al., 2000). Thirty microliters of the formic acid extract were mixed with cold orthophthaldehyde buffer solution (3 mM *o*-phthalaldehyde, 10 mM β-mercaptoethanol, and 100 mM potassium phosphate buffer, pH 6.8), incubated at 80°C for 15 min, and immediately cooled down on ice. The fluorescence was measured at 470 nm with an excitation at 410 nm using a fluorescence spectrophotometer (F2000 Hitachi, Tokyo, Japan).

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